

Short Communication

Toxicity of Alzheimer's disease-associated A β peptide is ameliorated in a *Drosophila* model by tight control of zinc and copper availability

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Abstract

Amyloid plaques consisting of aggregated A β peptide are a hallmark of Alzheimer's disease. Among the different forms of A β , the one of 42aa length (A β 42) is most aggregation-prone and also the most neurotoxic. We find that eye-specific expression of human A β 42 in *Drosophila* results in a degeneration of eye structures that progresses with age. Dietary supplements of zinc or copper ions exacerbate eye damage. Positive effects are seen with zinc/copper chelators, or with elevated expression of MTF-1, a transcription factor with a key role in metal homeostasis and detoxification, or with human or fly transgenes encoding metallothioneins, metal scavenger proteins. These results show that a tight control of zinc and copper availability can minimize cellular damage associated with A β 42 expression.

Keywords: Alzheimer's disease; amyloid- β protein; copper; heavy metal stress; MTF-1; zinc.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by amyloid plaques and neurofibrillary tangles in the brain. The main component of amyloid plaques, amyloid β peptide (A β), is widely regarded as the primary culprit in Alzheimer's disease (Selkoe, 2001). A β occurs in various lengths, whereby A β 42 is the most aggregation-prone and also the most neurotoxic form (Simmons et al., 1994; Gong et al., 2003; Hsieh et al., 2006; Shankar et al., 2007; Selkoe, 2008; Harmeier et al., 2009). The monomeric form of A β is not toxic (Selkoe, 2008) or at least less toxic than aggregated low-n multimers (Kayed et al., 2003; Selkoe, 2008; Harmeier et al., 2009). Amyloid plaques are highly enriched for zinc, copper and iron ions (Lovell et al., 1998), and histidines at positions 6, 13 and 14 of A β were shown to bind Zn(II) and Cu(II) (Atwood et al., 2000; Danielsson et al., 2007). Cell-

free studies have revealed that binding of Cu(II) or Zn(II) promotes the aggregation of synthetic A β (Atwood et al., 1998; Bush et al., 1994). Also, metal chelators can dissolve the A β plaques from postmortem AD brains (Cherny et al., 1999). A β -copper complexes were shown *in vitro* to generate reactive oxygen species (ROS) via a Fenton-like reaction (Huang et al., 1999). The fact that A β 42 in this assay has a higher propensity to generate ROS than A β 40 could, at least in part, explain why A β 42 is more neurotoxic and would suggest a role for redox-active metals such as copper and iron in this process. However, AD patients were found to have decreased copper levels in the cortex (Maurer et al., 2000; Cottrell et al., 2001) and in a mouse model of AD, copper supplementation reduced amyloid plaque formation and increased the activity of the antioxidant enzyme Cu/Zn SOD (Bayer et al., 2003).

In recent years, *Drosophila* models for Alzheimer's disease were developed (Finelli et al., 2004; Greeve et al., 2004; Crowther et al., 2005), and several factors (such as insulin-degrading enzyme, apolipoprotein E, oxidative stress) known to be related to Alzheimer's disease were studied with these *Drosophila* models (Rival et al., 2009; Sarantseva et al., 2009; Sofola et al., 2010; Tsuda et al., 2010; Ling and Salvaterra, 2011). Work from these groups has shown that *Drosophila* recapitulates several pathological features also seen in Alzheimer's disease. Based on this knowledge we generated a *Drosophila* model for AD to test various genetic and environmental factors that might affect the toxicity of A β 42.

To this end, we made transgenic flies expressing human A β 42. Peptide expression levels and signal peptide processing were verified by Western blot and mass spectrometry (Figure 1C). A β 42 expressed under the control of an eye-specific driver (GMR-Gal4) caused a degeneration of eye structure. The severity of the eye phenotype correlated with the A β 42 expression level and the age of the flies, i.e., when the expression level was higher or the flies grew older, eye malformations were more pronounced (Figure 2). The effect of zinc and copper ions on A β 42 toxicity was tested by raising or maintaining flies in food enriched for these essential metals. Based on previous experiments, 4 mM zinc or 500 μ M copper were chosen. These concentrations, which are relatively high but still tolerated by the standard fly strain (yw), consistently enhanced the eye distortion phenotype (Figure 3C, D). To find out whether the effect of zinc and copper on A β 42 toxicity was direct or indirect, the three histidines in A β 's copper/zinc binding site (positions 6, 13

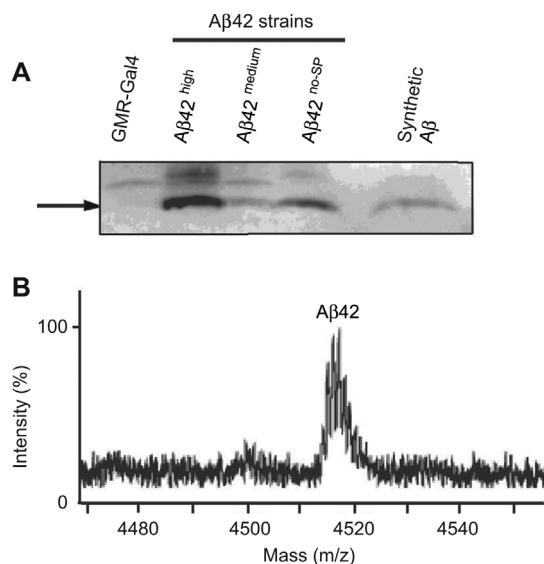


Figure 1 Expression of Aβ42 in *Drosophila*.

(A) Western blot with Aβ-specific antibody to detect Aβ42 in transgenic fly strains. Aβ42 peptides were extracted from transgenic fly lines with moderate or high Aβ42 expression levels (Aβ42-medium and Aβ42-high). The N-terminal signal peptide is correctly processed because it runs at the same position as the one from flies expressing Aβ42 without signal peptide, as evident from transgenic line Aβ42-no-SP and the synthetic Aβ42 controls (arrow). (B) Mass spectrometry of protein extract from UAS-Aβ42-high/+; GMR-Gal4/+ flies. In accordance with the Western blot in (A), the molecular weight of the peak peptide corresponds to Aβ42 without signal peptide.

Methods: flies were raised at 25°C and 65% humidity in standard food containing, per liter, 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar, and 15 ml anti-fungal agent nipagin (15% in ethanol, m/v). The Aβ42 coding sequence with the signal peptide sequence of the *Drosophila* hedgehog protein was cloned into the P element transformation vector pUAST. The sequence of Aβ42 with the signal peptide is: mdnhssvpwasaavtclslldakchssssssssksaaisaipqeetqtmrhahtqrcslrtslvalllvpvmvfspahsDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA, whereby lower case and capital letters denote signal peptide and Aβ42 sequences, respectively. To obtain strains UAS-Aβ42-high, UAS-Aβ42-medium, and UAS-hMT3, flies were transformed by standard P element transposition. Flies expressing Aβ42 without signal peptide (UAS-Aβ42-no-SP) were generated in the same manner. The phage C31 site-specific integration system was used to introduce sequences coding for Aβ42, or mutant Aβ42 with three histidines (His 6, 13 and 14 of the metal binding site) were changed into arginines. Wild type and mutant Aβ42 were cloned into an attB vector containing the 5xUAS enhancer/promoter and introduced into the specific attP 'landing site' of strain ZH-86Fb, resulting in transgenic flies UAS-Aβ-attB and UAS-Aβ-3HR-attB. To direct Aβ42 expression to the nerve system, the Gal4 driver was expressed from the elav enhancer/promoter, obtained from the Bloomington Stock Center.

Protein was isolated from control flies and Aβ42 expressing flies with RIPA buffer (50 mM Tris-HCl/0.5% sodium deoxycholate/1% NP-40/150 mM sodium chloride/1% SDS). Sepharose G beads were preincubated with WO-2 antibody directed against Aβ before being used for immunoprecipitation of Aβ peptides from the protein lysate. After immunoprecipitation, samples were loaded on a Novex 10%–20% Tricine gradient gel. For Maldi-MS, after immunoprecipitation, samples were washed twice with PBS and then washed twice with 50% ammonium acetate. Elution was done with 25% ammonium hydroxide. Maldi-MS was performed as previously described (Harmeier et al., 2009).

and 14) were replaced by arginines. Expression of such mutant Aβ42 caused only a mild eye degeneration, which could not be exacerbated by zinc supplement (Figure 3G, H). One might argue that the milder phenotype was merely due to a lower expression level. We cannot exclude this but consider it unlikely for two reasons. First, thanks to site-specific integration, both wild type and mutant(s) were inserted at the same chromosomal locus and driven by the same promoter, thereby eliminating differences due to chromosomal position effects. Second, the phenotype of the mutant was unaffected by zinc supplement, which argues for a specific and direct metal effect on Aβ42.

Systemic expression of Aβ42 under the control of a ubiquitous driver (actin-Gal4) decreased the overall survival rate of the flies (Figure 4A). Viability was further reduced by zinc supplement, but not by copper supplement or copper depletion, indicating that from the two metals, a body-wide surplus of zinc has more severe consequences. To further test the effect of metal concentration, we supplemented the food with metal chelators, notably DP-109 and DP-460 of the family of 'membrane-activated chelators' (MACs). By attaching to the cell membrane, MACs avoid the problems that could be caused by a general chelation of metals, and they were shown to reduce amyloid pathology in Tg2576 mice (Lee et al., 2004; Kolusheva et al., 2005; see also Petri et al., 2007). Indeed we found that DP-109, a zinc/copper chelator, increased the fraction of Aβ42-expressing flies developing to adulthood (Figure 4A).

With eye-specific Aβ42 expression, eye damage was exacerbated by both zinc and copper supplement but ameliorated by DP-109 or BCS administration (Figure 4B–E). In control flies not expressing Aβ42, normal eye structure was not affected by copper or zinc treatment (data not shown). When the expression of Aβ42 was directed to the nerve system by using a neuron-specific driver (elav-Gal4) the flies exhibited defects in locomotion which were more severe upon zinc supplementation, as measured by a standard climbing assay:

the number of control flies able to climb the vertical distance within a given time fell from 100% at day 1 to 92% at day 5 to 72% at day 10. At the same time, the number of successful flies on food with 4 mM zinc fell dramatically from 80% to 33% to 3%. Under these latter conditions, there was a strong rescue effect with a MAC-type chelator (Figure 4F). The results with mice mentioned above and our own ones indicate that MACs can efficiently counteract the deleterious effects of metals in conjunction with Aβ. Of note, upon systemic expression of Aβ42, DP-109 was effective in the survival assay whereas the copper-specific chelator BCS was ineffective. This suggests that DP109's major role under this condition was the chelation of zinc.

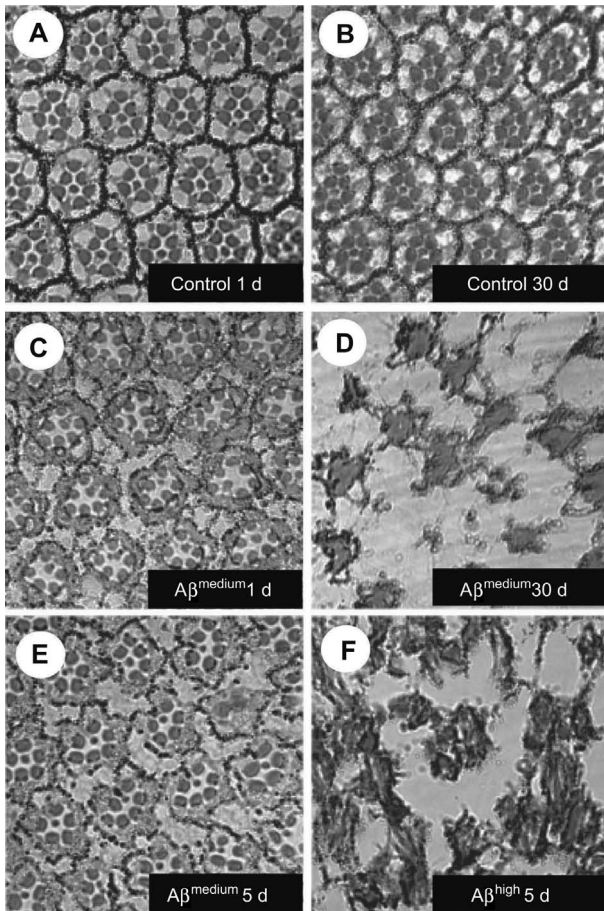


Figure 2 A β 42 expression in the eye causes progressive eye degeneration correlated with age and expression levels.

While the solid surface of the complex eyes remained largely intact, sections revealed a progressive decay of ommatidial organization in A β 42-expressing vs. control flies: controls expressing only the GMR-Gal4 driver maintained normal structures either in young (1-day) (A) and old (30-day) flies (B). A β -dependent eye degeneration was more severe in 30-day-old flies (D) than in 1-day-old flies (C). (E) and (F) show eye sections of 5-day-old flies expressing A β 42 either moderately (UAS-A β -medium/+; GMR-Gal4/+), or at high level (UAS-A β -high/+; GMR-Gal4/+).

Methods: unless specified otherwise, adult flies were collected at 5 days of age and eye sections were analyzed with a Leica Leitz DMRB fluorescence stereomicroscope (Leica, Wetzlar, Germany). Note that complex eyes were not always cut at the very same level, hence even the contours of intact ommatidia may show some variation in this and the following Figures; nevertheless, a distorted ommatidial structure was always evident irrespective of the plane of section.

In every organism, intracellular zinc and copper levels have to be carefully controlled to avoid starvation or toxic effects from overload. The zinc finger transcription factor MTF-1 (metal responsive transcription factor, or metal response element binding transcription factor), is a key regulator of heavy metal homeostasis and metal detoxification from insects to mammals (Radtke et al., 1993; reviewed in Andrews, 2001; Lichtlen and Schaffner, 2001; Egli et al.,

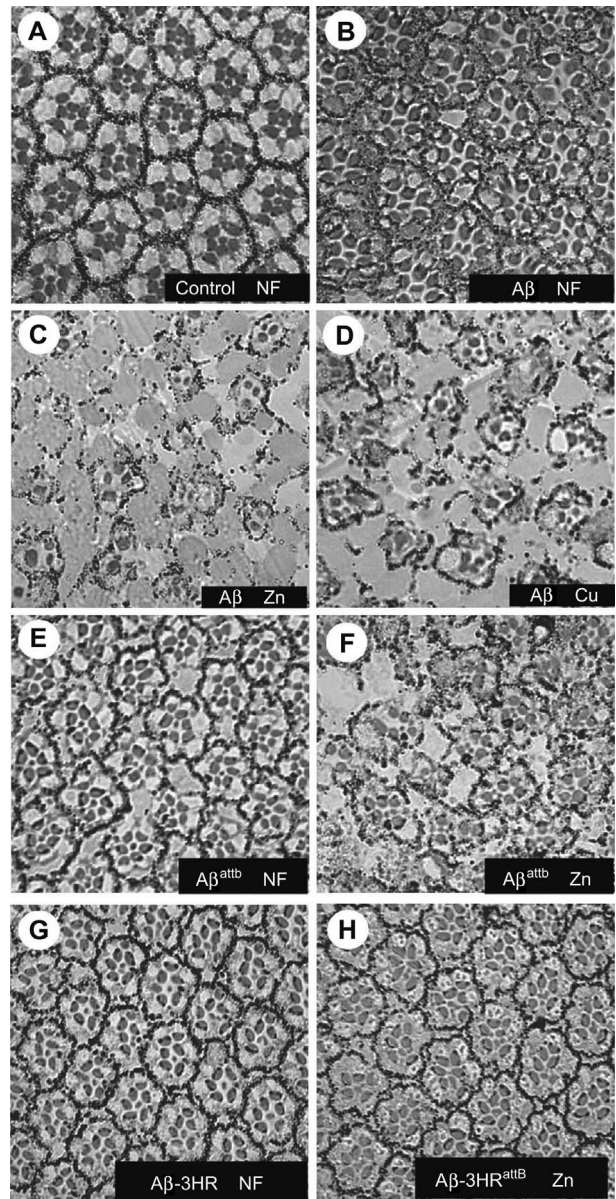


Figure 3 Zinc or copper food supplement exacerbates eye distortion.

Adult flies that had been kept in different food for 5 days after eclosure from the puparium were collected and eye sections analyzed as described in Figure 2. These include control flies (GMR-Gal4/+) grown on standard food (NF) (A) or flies expressing A β 42 in the eyes (UAS-A β S3/+; GMR-Gal4/+), also grown on standard food (B) or on food containing 4 mM ZnCl₂ (C) or 500 μ M CuSO₄ (D). Flies expressing A β 42 (E, F) or mutant A β 42 with 3His \rightarrow Arg substitutions to abolish metal binding (G, H) were grown on either standard food (NF) (E, G) or food containing 4 mM Zn (F, H).

Methods: for experiments, food was supplemented to the indicated concentrations with either CuSO₄, ZnCl₂, the copper chelator bathocuproinedisulfonate (BCS) disodium salt hydrate (Sigma-Aldrich No. 14,662-5) or 'membrane activated chelators' DP-109 and DP-460 (D-Pharm, Rehovot) to the indicated concentrations. For simplicity, the ionic metal compounds are in most cases referred to as zinc (Zn) or copper (Cu).

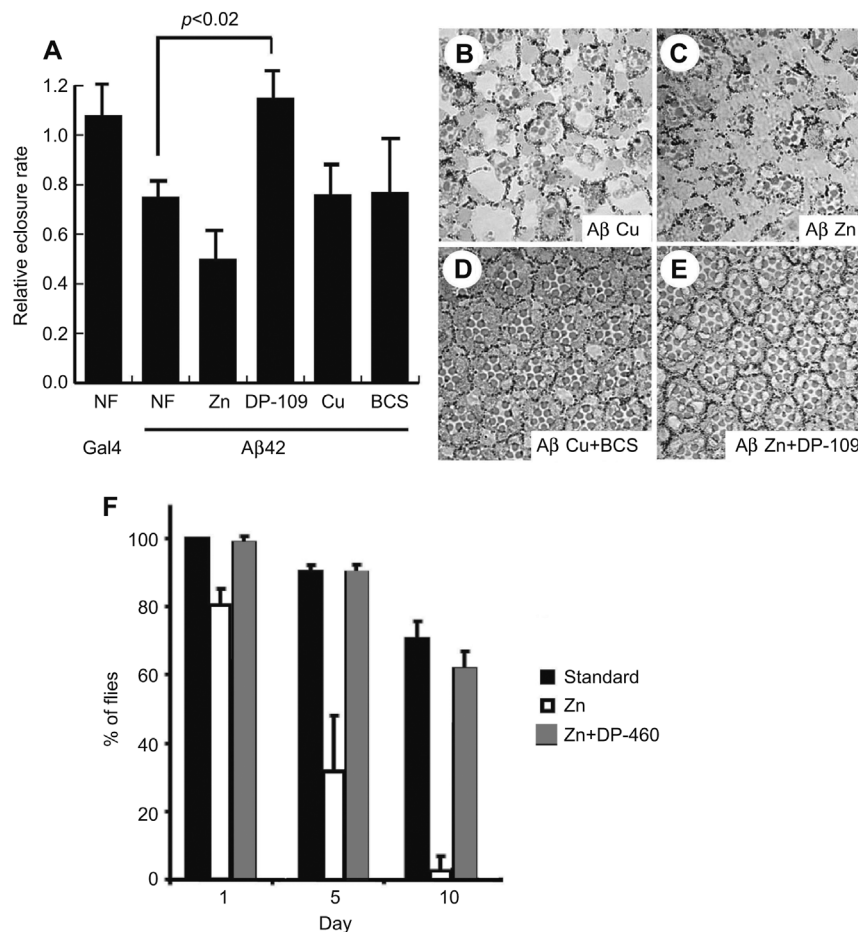


Figure 4 Metal chelators ameliorate Aβ42-mediated phenotypes.

(A) Survival assay using control flies and Aβ42-expressing flies. Ubiquitous expression of Aβ42 via actin-Gal4 reduced viability, evident as a lower eclosure rate (flies completing development to adulthood). The decline in survival was counteracted by supplementation with the Zn/Cu chelator DP-109 but not by supplementation with BCS chelator which preferentially binds Cu(I). (B–E) Eye sections of 5-day-old UAS-Aβ42-medium/+; GMR-Gal4/+ flies raised and kept on food containing 500 μM Cu (B) or 4 mM Zn (C); on food containing both 500 μM Cu and 250 μM BCS (D), or both 4 mM Zn and 100 μM DP-109 (E). (F) Aβ42 expression in the nerve system affects motoric coordination ability, as measured by a climbing assay with UAS-Aβ42 high/elav-Gal4 flies that were raised and kept on standard food (NF), on food containing 4 mM Zn, or food containing 4 mM Zn in combination with 100 μM chelator DP-460.

Methods: the survival index was calculated based on the eclosure rate of the flies (i.e., flies completing development to adulthood). Flies that carry a UAS-Aβ42S7 transgene were crossed with actin-Gal4/TM3, y+ flies on standard food or on food containing either Cu, Zn, DP-109 or BCS. From the cross, two types of progeny could be obtained, namely, (a) flies that were expressing Aβ42 or (b) control flies that did not express Aβ42. The survival index (Is) was calculated as follows: $Is = 2a/(a+b)$. For the climbing assay, groups of female flies were transferred into empty 95×27 mm glass tubes which were marked with a horizontal line 7.5 cm above the bottom. Flies were gently shaken down to the bottom of the vial and after 10 s the number of flies that had climbed beyond the 7.5 cm mark was recorded. All the locomotor studies were performed under standardized light conditions. Treatment with MACs does not interfere with the uptake of zinc and copper in flies (data not shown).

2003; Rutherford and Bird, 2004; Balamurugan and Schaffner, 2006). A major class of MTF-1 target genes encodes metallothioneins, cysteine-rich scavenger proteins for a number of heavy metals. MTF-1 is also involved in the cellular defense against other stress conditions, including oxidative stress and hypoxia. While the lack of MTF-1 renders flies sensitive to metals, MTF-1 overexpression confers enhanced protection against heavy metal stress (Balamurugan et al., 2007). Also in our model system, elevated expression of MTF-1 or of one of its major target genes, metallothionein A (MtnA),

strongly ameliorated Aβ toxicity (Figure 5C, D). Conversely, in an MTF-1 null mutant background, Aβ exerted more severe damage to the eye tissue (Figure 5B), corroborating the influence of MTF-1 on the Aβ42 phenotype. We also tested human metallothionein 3 (MT3), the most abundant metallothionein in the brain, in our system. Indeed the human MT3 transgene co-expressed with Aβ42 suppressed the Aβ-mediated phenotype (Figure 5E). Although it is still unclear whether reduced levels of metallothioneins, notably of the brain-enriched MT3, contribute to AD (Uchida et al., 1991;

Erickson et al., 1994; Uchida, 1994), our results are consistent with earlier findings that metallothioneins have neuroprotective functions (Irie and Keung, 2003; Penkowa, 2006; Meloni et al., 2008; West et al., 2008).

We have previously shown that flies lacking Parkin, a ubiquitin ligase involved in Parkinson's disease, also benefit from a tight control of copper availability and upregulation of antioxidant response (Saini et al., 2010). In line with the findings presented here, elevated expression of MTF-1 dramatically improved the condition of the *parkin* mutant (Saini et al., 2011). However, unlike the A β transgenics described here where extra zinc was at least as bad, if not worse than copper, zinc supplementation had a strong beneficial effect on the *parkin* mutant (Saini and Schaffner, 2010). Taken together, these findings underline the importance of metals as modulators in pathogenic processes, notably ones that are

related to human neurodegenerative diseases, but they also indicate specific requirements depending on the condition.

A large body of evidence indicates that oxidative stress contributes to AD; for example, patients have higher concentrations of oxidative stress markers than age-matched controls (Markesbery and Lovell, 1998). A previous study suggested that chelation of copper reduces A β -mediated hydrogen peroxide generation and oxidative damage (Huang et al., 1999). To investigate how oxidative stress affects A β 42 toxicity, we treated A β 42-expressing flies with hydrogen peroxide. We found that at a dosage that does not affect wild type flies, hydrogen peroxide strongly enhances the eye degeneration phenotype in A β 42 transgenic flies (Figure 6).

In our *Drosophila* model, not only zinc excess but also elevated copper availability enhances the toxicity of A β . The latter would be in line with a role of copper in the generation of ROS by redox cycling, but at variance with some findings in mammalian systems, where copper is known to be involved at multiple levels. Copper also binds to APP and BACE (beta-secretase) and modulates APP processing and A β peptide production (Simons et al., 2002; Barnham et al., 2003; Angeletti et al., 2005). In the brains of AD patients, copper homeostasis is disturbed, whereby copper is enriched in the amyloid plaques but its overall level is decreased, resulting in a reduced Cu/Zn SOD-1 activity (Lovell et al., 1998). In a transgenic mouse model of AD, dietary copper restored the function of SOD-1 and reduced plaque formation (Bayer et al., 2003). This somewhat counter-intuitive beneficial effect of copper prompted a one-year clinical

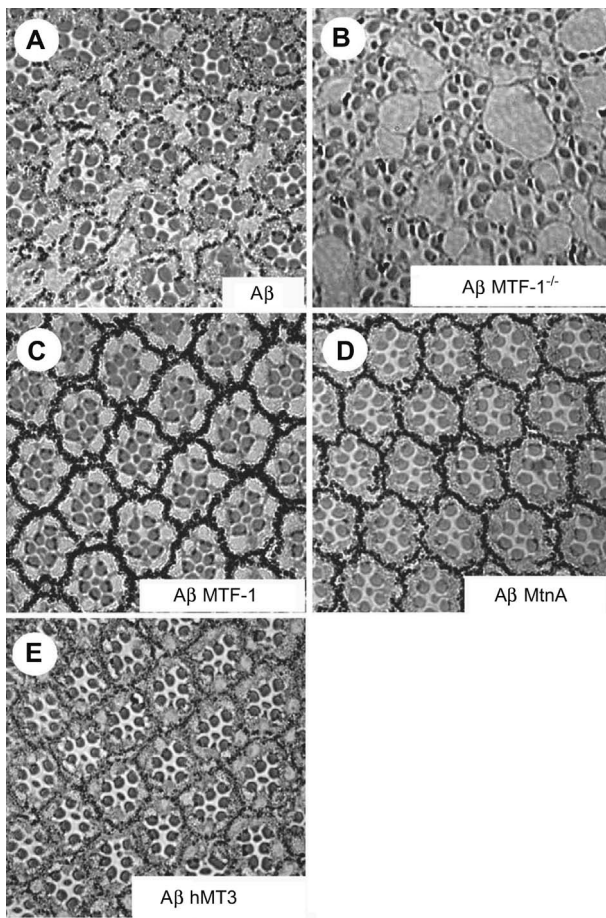


Figure 5 MTF-1 and metallothioneins ameliorate A β 42-mediated eye degeneration.

Shown are eye sections of 5-day-old flies expressing A β 42 alone (A) or flies co-expressing A β 42 and the key transcription factor for heavy metal homeostasis and detoxification, MTF-1 of *Drosophila* (C) the major *Drosophila* metallothionein MtnA (D) or human brain-specific metallothionein MT3 cloned into the pUAST vector (E) MTF-1, MtnA and human MT3 transgenes were under the regulation of UAS (upstream activation sequence) promoter. (B) Eye phenotype of A β 42 expression in flies lacking the metal responsive regulator MTF-1.

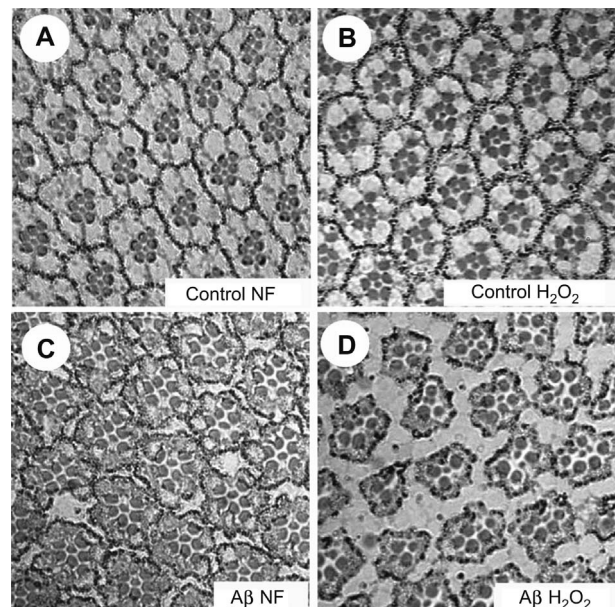


Figure 6 A β 42-expressing flies, but not control flies, are hypersensitive to hydrogen peroxide.

The eyes of control flies (GMR-Gal4/+) displayed no obvious difference between standard food (A) and food containing 0.025% H₂O₂ (B) However, flies expressing A β 42 treated with 0.025% H₂O₂ (D) showed stronger eye degeneration than those grown on standard food (C).

study with copper supplementation (8 mg/day) to patients with mild AD. This study did not yield conclusive results in terms of amelioration of cognitive performance but also had no negative effects (Kessler et al., 2008a,b). Based on the original idea that copper might exacerbate AD progression, the metal chelator clioquinol was shown to decrease A β deposition in Tg2576 transgenic mice (Cherny et al., 2001) and in a phase II clinical trial was reported to have beneficial effects. A phase III clinical trial termed PBT2 (see <http://www.alzforum.org/drg/drc/detail.asp?id=110>) is under way. However, even if clioquinol turns out to counteract AD, it might not act by merely scavenging copper, but rather to function as a copper carrier to effectively increase intracellular copper levels, as was shown in a yeast model (Treiber et al., 2004). Thus, while elevated zinc levels promote A β aggregation and have adverse effects *in vivo*, the role of copper remains to be elucidated.

In conclusion, we show that the toxicity of A β peptides can be reduced either by chelation of aggregation-promoting or redox-active metal ions (Zn, Cu), or by reducing oxidative stress. In addition to metal chelators, we find that expression of the key regulator of metal homeostasis, MTF-1, or of human or *Drosophila* metallothioneins is also capable of reducing A β phenotypes. Since MTF-1 is involved in the defense against several cell stress conditions and is effective in *Drosophila* models of Parkinson's and Alzheimer's diseases (Saini et al., 2011 and shown here), it would be of interest to determine if polymorphisms/genetic variations in MTF-1 gene expression modulate the course of AD and other human neurodegenerative disorders. Also, further investigations on metal-ion mediated effects, such as those described here, are expected to shed more light on the molecular mechanisms of AD pathogenesis.

Acknowledgments

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Conflict of interest statement

MAC samples were kindly provided by D-Pharm Ltd. D-Pharm had no role in the experimental design, in analyzing the results, or in the preparation of the manuscript.

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